

AD _____

Award Number: DAMD17-01-1-0332

TITLE: Expression of Metabolic and Apoptotic Genes During
Treatment With Chemopreventive Agents for Breast Cancer

PRINCIPAL INVESTIGATOR: Xuexian Zhang, Ph.D.

CONTRACTING ORGANIZATION: University of Minnesota
Minneapolis, Minnesota 55455-2070

REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20021127 085

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)

2. REPORT DATE

July 2002

3. REPORT TYPE AND DATES COVERED

Annual Summary (1 Jul 01 - 30 Jun 02)

4. TITLE AND SUBTITLE

Expression of Metabolic and Apoptotic Genes
During Treatment With Chemopreventive Agents for
Breast Cancer

5. FUNDING NUMBERS

DAMD17-01-1-0332

6. AUTHOR(S)

Xuexian Zhang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Minnesota
Minneapolis, Minnesota 55455-2070

E-mail: zhang112@tc.umn.edu

8. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information)

Effects of short-term (up to 2 weeks) treatment of rats with indole-3-carbinol (I3C) at three dose levels on mRNA expression of cytochrome P450 (CYP) in the liver and mammary gland and apoptotic activity in the mammary gland were examined. The mRNA transcripts for hepatic CYP1A1, 1B1, and 2B1/2 and mammary CYP1A1 were upregulated after treatment with I3C at 250 mg/kg of body weight. This treatment also increased the oxidative metabolism of 17 β -estradiol (E2) and estrone (E1) by liver microsomes. In the mammary gland, activities of caspase-3, -8, and -9 were induced by I3C at lower dose levels (5 mg/kg for 4-dose and 25 mg/kg for 10-dose treatment). These results show that treatment with I3C at the high dose level altered the CYP complement and metabolite composition from E2 and E1, and that at the lower dose levels induced apoptosis in tumor-target organ. The data suggest that mechanism(s) of induction of apoptosis by I3C does not involve modulation of P450-dependent estrogen mechanism. Likewise, apoptotic activity was not induced in mammary tumors (adenocarcinomas) during post-carcinogen treatment of rats with I3C at 250 mg/kg (24 to 36 doses during 8-12 weeks). The level of apoptosis in mammary tumors was independent of treatment and likely reflected the intrinsic process of tumor apoptosis.

14. SUBJECT TERMS

chemoprevention, apoptosis, CYP450, DMBA, indole-3-carbinal, RT-PCR,
tamoxifen, breast cancer

15. NUMBER OF PAGES

6

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT

Unclassified

18. SECURITY CLASSIFICATION
OF THIS PAGE

Unclassified

19. SECURITY CLASSIFICATION
OF ABSTRACT

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	
SF 298.....	
Objective.....	1
Studies and Results.....	1
Publication.....	2
Appendices.....	2

1. OBJECTIVE

Indole-3-carbinol (I3C), a compound found in cruciferous vegetables, has been shown to suppress estrogen-dependent cancers. Experiments are aimed at identifying the metabolic and apoptotic genes underlying the mechanisms of action of I3C in experimentally induced mammary carcinogenesis. The specific aims include determination of: 1) the effects of short-term (up to 2 weeks) treatment of rats with I3C at three dose levels (5, 25, and 250 mg/kg body weight (bwt)) on mRNA expression of cytochrome P450s (CYPs) in the liver and mammary gland and apoptotic activities in the mammary gland; and 2) the effects of long-term (up to 16 weeks) post-carcinogen treatment of rats with I3C and/or tamoxifen (TAM) on mammary tumorigenesis and apoptosis in the mammary gland and mammary tumors.

2. STUDIES AND RESULTS

Under aim 1, treatments of female Sprague-Dawley rats included three dose levels of I3C, i.e. 5, 25, and 250 mg/kg of bwt, administered by oral gavage in 20% ethanol in olive oil for 4 and 10 days. Rats were sacrificed 24 hrs after the last dose, and liver and mammary glands were collected and stored at -80°C . CYP mRNA expression in the liver and mammary gland, CYP probe activities, and CYP-dependent metabolism of 17β -estradiol (E2) and estrone (E1) by liver microsomes were examined. The methods included: semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) to estimate CYP mRNA transcripts, spectrophotometric assay of P450:carbon monoxide complex, spectrofluorimetric assays of CYP specific alkoxyresorufin *O*-dealkylase activities, and HPLC/radiometric assays of metabolites from ^{14}C -labeled E2 and E1. The mRNA transcripts for hepatic CYP1A1, 1B1, and 2B1/2 and mammary CYP1A1 were upregulated after treatment with I3C at 250 mg/kg. This treatment also increased the capacity of liver microsomes to metabolize E2 to 2-OH-E2, 2-OH-E1, 6- α -OH-E2, 6- β -OH-E2, estriol and 15- α -OH-E2, and E1 to 2-OH-E1, 2-OH-E2, 6(α + β)-OH-E1 and 6 α -OH-E2. The increases of CYP-dependent activities and rates of estrogen metabolite formation were smaller after 10 than 4 treatments. The results indicate that alterations in CYP complement and metabolite composition from E2 and E1 are I3C dose- and treatment duration-dependent and suggest that biological activity of I3C administered at low dose levels does not involve the changes in estrogen metabolism.

Further, apoptosis in the mammary gland after the 4- and 10-day treatments of rats with I3C at the above dose levels was examined. Total protein was isolated from mammary gland, quantitated, and used to measure the activities of caspases with specific substrates, *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide for caspase-3, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide for caspase-8, and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide for caspase-9. The relative absorbance at 405 nm was determined on plate reader. Statistical analysis was performed with ANOVA. Four-dose treatment with I3C at 5 mg/kg bwt induced significantly ($P < 0.05$) the activities of caspase-3 (3.3-fold), caspase-8 (2.5-fold), and caspase-9 (2.5-fold). After 10-dose treatment with I3C at 25 mg/kg bwt, the activities of caspase-3 (3.6-fold), caspase-8 (1.8-fold), and caspase-9 (2-fold) were induced significantly ($P < 0.05$). To elucidate and confirm dose-related effects of I3C on caspase activities, other apoptotic genes (e.g. PARP, DFF45) will be assayed to determine the mechanisms by which I3C may affect mammary gland before and during carcinogenesis process.

Under aim 2, The methods for assays of apoptotic activities in mammary tumors were evaluated using the tissues from the Mentor's previous study (*Cancer Letters* 160: 209-218, 2000). Thus, apoptotic activities in 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary adenocarcinomas in female Sprague-Dawley rats, and potential modulation of apoptosis by treatment of rats with indole-3-carbinol (I3C) at 250 mg/kg or β -naphthoflavone (β -NF) at 20 mg/kg of body weight in ethanol:corn oil (2:3), by oral gavage, three times per week for up to 12 weeks starting 3 weeks after DMBA (one oral dose of 20 mg per 7-week-old rat) were examined. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed in tumor sections prepared from paraffin-embedded tissues. The number of apoptotic cells and total number of cells in each field (6 to 8 fields per section) were counted using light microscopy and Image-Plus program. The percentages of apoptotic cells were $2.95 \pm 1.23\%$ ($n = 10$), $4.93 \pm 3.31\%$ ($n = 12$), and $3.61 \pm 1.72\%$ ($n = 11$) in tumors from the vehicle-, I3C- and β -NF-treated rats, respectively. Caspase-3, caspase-8, and caspase-9 activities were determined with *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide, *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide as the specific substrate, respectively, using total tumor protein and colorimetric (absorbance at 405 nm) development. The relative absorbance for caspase-3 activity was 0.306 ± 0.154 ($n = 16$) for vehicle-, 0.321 ± 0.158 ($n = 20$) for I3C- or 0.372 ± 0.136 ($n = 15$) for β -NF-treated rats. However, the higher activities of caspase-3 in tumors of β -NF- or I3C-treated rats were not significantly different from that of the vehicle-treated group ($P = 0.11$). Likewise, the relative absorbances for caspase-8 activity, 0.225 ± 0.083 ($n = 12$) for vehicle-, 0.178 ± 0.090 ($n = 12$) for I3C- or 0.255 ± 0.075 for β -NF-treated rats, and caspase-9 activity, 0.152 ± 0.100 ($n = 10$) for vehicle-, 0.175 ± 0.075 ($n = 8$) for I3C- or 0.243 ± 0.105 ($n = 8$) for β -NF-treated rats were not significantly different among the groups. Protein expression levels of the antiapoptotic gene, Bcl-2, and apoptotic gene Bax, were determined by Western blot analysis using total tumor protein. The pattern of expression of Bcl-2 or Bax was variable, and it did not follow the reported upregulation of Bax by I3C in breast cancer cells *in vitro*. Although I3C or β -NF administered before DMBA suppresses mammary tumorigenesis, these compounds in the post-carcinogen regimen do not significantly change the outcome of tumorigenesis and apoptotic activities in mammary tumors.

3. PUBLICATION

Abstracts:

- 1) Zhang, X. and Malejka-Giganti, D. Detection of apoptosis in rat mammary adenocarcinomas. In: Era of Hope Department of Defense Breast Cancer Research Program Meeting, Orlando, FL, Sep 25-28, 2002.
- 2) Beland, F.A., Churchwell M.I., Doerge, D.R., Malejka-Giganti, D., Zhang, X., Divi R.L., Poirier, M.C., Costa, G.G., Marques, M.M. High performance liquid chromatography electrospray ionization tandem mass spectrometry analysis of tamoxifen DNA adducts in rats, monkeys, and humans. *Proc Amer Assoc Cancer Res.* 43: 343,2002.

4. APPENDICES

Abstract for DoD Meeting: "DETECTION OF APOPTOSIS IN RAT MAMMARY ADENOCARCINOMAS "

DETECTION OF APOPTOSIS IN RAT MAMMARY ADENOCARCINOMAS

Xuexian Zhang and Danuta Malejka-Giganti

Veterans Affairs Medical Center, Research Service (151), One Veterans Drive, Minneapolis, MN 55417, Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455

Induction of apoptosis is one approach to suppression of cancer. Extensive efforts have focused on induction of apoptosis in cancer cells *in vitro* and elucidation of the mechanisms of apoptosis by antitumor agents. However, data on drug-modulated apoptotic activities in animal tumor models are scarce. Our study examined apoptotic activities in 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary adenocarcinomas in female Sprague-Dawley rats, and potential modulation of apoptosis by treatment of rats with indole-3-carbinol (I3C) at 250 mg/kg or β -naphthoflavone (β -NF) at 20 mg/kg of body weight in ethanol:corn oil (2:3), by oral gavage, three times per week for up to 12 weeks starting 3 weeks after DMBA (one oral dose of 20 mg per 7-week-old rat). Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed in tumor sections prepared from paraffin-embedded tissues. The number of apoptotic cells and total number of cells in each field (6 to 8 fields per section) were counted using light microscopy and Image-Plus program. The percentages of apoptotic cells were $2.95 \pm 1.23\%$ ($n = 10$), $4.93 \pm 3.31\%$ ($n = 12$), and $3.61 \pm 1.72\%$ ($n = 11$) in tumors from the vehicle-, I3C- and β -NF-treated rats, respectively. Caspase-3 and caspase-9 activities were determined with *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide as the specific substrate, respectively, using total tumor protein and colorimetric (absorbance at 405 nm) enzyme-linked immunosorbent assays (ELISA). The relative absorbance for caspase-3 activity was 0.306 ± 0.154 ($n = 16$) for vehicle-, 0.321 ± 0.158 ($n = 20$) for I3C- or 0.372 ± 0.136 ($n = 15$) for β -NF-treated rats. However, the higher activities of caspase-3 in tumors of β -NF- or I3C-treated rats were not significantly different from that of the control group ($p = 0.11$). Likewise, the relative absorbance for caspase-9 activity of 0.152 ± 0.100 ($n = 10$) for vehicle-, 0.175 ± 0.075 ($n = 8$) for I3C- or 0.243 ± 0.105 ($n = 8$) for β -NF-treated rats was not significantly different among the groups. Protein expression levels of the antiapoptotic gene, Bcl-2, and apoptotic gene Bax, were determined by Western blot analysis using total tumor protein. The pattern of expression of Bcl-2 or Bax was variable, and it did not follow the reported upregulation of Bax by I3C in breast cancer cells *in vitro*. In contrast to inhibition of mammary tumorigenesis by I3C or β -NF administered before DMBA, these compounds used in the post-carcinogen regimen do not significantly change the outcome of tumorigenesis observed in our previous study and apoptotic activities in mammary tumors shown herein.

The U.S. Army Medical Research Materiel Command under DAMD17-01-1-0332 supported this work.